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Characterization of Cleavage and Polyadenylation Specificity Factor and Cloning of Its 100-Kilodalton Subunit

ANDREAS JENNY, HANS-PETER HAURI, AND WALTER KELLER*

Department of Cell Biology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

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During the formation of the 3′ ends of mRNA, the cleavage and polyadenylation specificity factor (CPSF) is required for 3′ cleavage of the transcript as well as for subsequent polyadenylation. Using peptide sequences from a tryptic digest, we have cloned the 100-kDa subunit of CPSF. This subunit is a novel protein showing no homology to any known polypeptide in databases. Polyclonal antibodies against the C terminus of the protein inhibit the polyadenylation reaction. Polyclonal and monoclonal antibodies were used to characterize the composition of CPSF. Immunoprecipitations of CPSF from HeLa cell extracts and from labeled chromatographic fractions show the coprecipitation of all four subunits of 160, 100, 73, and 30 kDa. Proteins of 160 and 30 kDa that are specifically cross-linked to precursor RNA by UV irradiation were identified as CPSF subunits by immunoprecipitation. Immunofluorescent detection of CPSF in HeLa cells localized it in the nucleoplasm, excluding cytoplasm and nucleolar structures.

Most mRNAs in eukaryotes receive a poly(A) tail posttranscriptionally (reviewed in reference 42). This tail has important although not fully elucidated functions in mRNA stability and translation initiation (reviewed in references 11, 17, 31, and 32). The tail length of about 200 to 250 residues is in some cases critical for translational control (46). 3′ processing occurs in a three-step reaction. First, the primary transcript is cleaved endonucleolytically at the polyadenylation site, and then the upstream cleavage product slowly receives an oligo(A) tail of 10 to 12 nucleotides (34). In a third phase, the oligo(A) tail is elongated in a fast and processive reaction (2, 40).

The accuracy of the cleavage reaction depends on two sequence elements in the primary transcript (reviewed in reference 30). The AAUAAA polyadenylation signal is located 10 to 30 nucleotides upstream of the poly(A) site. It is highly conserved among vertebrates and very susceptible to mutations and modifications (1, 45). A downstream signal is less well defined and is usually referred to as a stretch of U- or G/U-rich residues. In vivo, 3′ cleavage and polyadenylation are tightly coupled, but in vitro, the cleavage reaction can be studied separately by blocking the poly(A) polymerase activity with EDTA or chain-terminating nucleotides (26). Biochemical fractionation revealed that in addition to poly(A) polymerase (7) at least three factors are involved in the cleavage process: cleavage and polyadenylation specificity factor (CPSF [3, 12, 27]), cleavage factors I and II (6, 38), and cleavage stimulation factor (CStF [37]). The oligoadenylation requires only CPSF and poly(A) polymerase, whereas the processive tail elongation additionally needs poly(A)-binding protein II (PABII) (2, 40). It is clear that CPSF has a central role in polyadenylation not only because it binds directly to the polyadenylation signal but also because it is involved in all three reaction steps.

Direct interactions with the RNA substrate were shown for CStF and CPSF. The 64-kDa subunit of CStF can be cross-linked by UV light to RNA substrates containing the AAUAAA sequence in a CPSF-dependent way (47, 48). The RNA binding of bacterially expressed 64-kDa subunit, however, no longer shows this specificity (36).

Purified CPSF consists of four polypeptides with molecular masses of 160, 100, 73, and 30 kDa (3). A more recent report (27) did not confirm the presence of the 30-kDa subunit in pure CPSF preparations. It was suggested that this subunit might be more loosely associated with the complex (24). Modification interference experiments have shown that CPSF is in direct contact with the polyadenylation signal (18). It was also shown that HeLa nuclear extracts and partially purified CPSF fractions contained proteins of 160 and 30 kDa which could be UV cross-linked to RNA when a functional polyadenylation signal was present (12, 18, 25). The identity of these proteins was controversial, since other studies could not detect UV-cross-linkable proteins with these molecular masses (27, 47).

In this study, we report cloning of the 100-kDa subunit of CPSF. Its predicted amino acid sequence is unrelated to any known protein sequence. Immunoprecipitation experiments with subunit-specific polyclonal and monoclonal antibodies suggest that all four proteins described above are subunits of CPSF and that the two UV-cross-linkable proteins are the 160- and 30-kDa subunits of CPSF. Furthermore, immunohistochemical staining of HeLa cells shows a nuclear staining pattern excluding nucleoli and cytoplasm.

MATERIALS AND METHODS

Trypsin and phenylmethylsulfonyl fluoride were from Boehringer Mannheim, mercaptopropionic acid was from Fluka, and Hybond N^+ membranes and radiochemicals were from Amersham, except [^{35}S]methionine, which was from ICN. *Pfu* DNA polymerase and the bovine liver cDNA library were from Stratagene, protein A-Sepharose was from Pharmacia, horseradish peroxidase-conjugated goat anti-mouse antibodies were from Cappel, and chloronaphthol and chloramine T were from Merck. Bovine poly(A) polymerase (41) was a gift from Elmar Wahle. Affinity-purified anti-PAB II antiserum was a gift from Sabine Krause (20).

Tryptic digestion and amino acid sequencing. Purified CPSF [poly(A)-Sepharose pool (3)] corresponding to 15 μg of 100-kDa subunit was dialyzed against 100 mM NH_4HCO_3–0.1%

* Corresponding author. Mailing address: Department of Cell Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland. Phone: 41-61-2672060. Fax: 41-61-2672078. Electronic mail address: Keller2@urz.unibas.ch.

sodium dodecyl sulfate (SDS), and dried. The pellet was resuspended in 1 ml of 100 mM Tris-HCl (pH 7.5)–2% SDS–10 mM dithiothreitol, boiled for 5 min, and cooled to room temperature. Cysteines were carboxymethylated by the addition of iodoacetamide to a final concentration of 50 mM and incubation for 45 min at room temperature in the dark. The CPSF subunits were separated on an SDS–9% polyacrylamide gel (21) with 1 mM mercaptopropionic acid in the running buffer. After staining of the gel for 5 min with Coomassie brilliant blue R250, the 100-kDa subunit was excised and electroeluted in 100 mM NH_4HCO_3–0.1% SDS (BioTrap, Schleicher and Schuell). The eluted protein was precipitated with 9 volumes of ethanol. The pellet was washed once with 100% ethanol, resuspended in 20 μl of 8 M urea–0.4 M NH_4HCO_3, and diluted with 60 μl of H_2O. After the addition of 0.6 μg of trypsin, the protein was incubated for 7 h at 37°C. Another 0.6 μg of trypsin was added, and the incubation was continued overnight. The digest was separated over an Aquapore RP-300 column (C_8; 2.1 by 250 mm), and single peptides were rechromatographed over a Spheri 5 RP-18 column (C_{18}, 1 by 50 mm). The peptides were sequenced (Paul Jenö, Biozentrum) on an Applied Biosystems 477A sequencer according to the procedures recommended by the manufacturer.

Isolation of cDNA clones and sequencing. Peptide T23-2 was used to generate a screening oligonucleotide (22) with the sequence 5′ CTG CAT GAG ACA GTG GAT GCI ACI RCI GAG ACC CAC ATC TAC CAG GTG CG 3′. A total of 5×10^5 PFU of a random- and oligo(dT)-primed bovine liver cDNA library in λZAPII was screened in duplicate at a density of 5×10^4 per 150-mm plate with Hybond N^+ filters. The oligonucleotide was labeled with [γ-^{32}P]ATP and polynucleotide kinase to a specific activity of 3×10^6 cpm/pmol and purified over a Sephadex G-25 spin column. Hybridization conditions were as described in reference 39 except that 1% SDS was included in the hybridization buffer. The concentration of labeled oligonucleotide in the hybridization was 0.4 pmol/ml. The filters were washed twice for 15 min at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS and then for 15 min at room temperature in 0.2× SSC–0.5% SDS. A single positive clone (A1) was plaque purified. Nucleotides encoding peptide T-23 were identified by sequencing. RNA purifications and Northern (RNA) blots were conducted according to standard procedures (33). A1 was used to screen a calf thymus plasmid cDNA library as described previously (33). Five positive clones were purified. The longest one was sequenced. Exo III deletions (16) were produced (Erase-a-Base system, Promega) and sequenced on an Applied Biosystems 373A sequencer with dye terminators according to the manufacturer's instructions. Every base pair was sequenced at least once in the sense and the antisense strand. DNA and translated protein sequences were analyzed with Genetics Computer Group software (8). Data libraries were searched with the programs FASTA and TFASTA (29).

Production of polyclonal and monoclonal antibodies. A cDNA fragment starting at peptide T23-2 and ending after the stop codon of the 100-kDa subunit (terminal third of the protein) was amplified with *Pfu* DNA polymerase, introducing *Bam*HI and *Sal*I linkers at the 5′ and 3′ ends, respectively. This fragment was cloned into the vector pQE11 (Diagen) behind a tag of six histidines. *Escherichia coli* M15 (Diagen) was transformed, and 500-ml Luria broth cultures were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at an optical density at 600 nm of 0.7 and grown for another 5 h at 37°C. The His-tagged fusion protein was purified over a Ni-nitrilotriacetic acid agarose (Diagen) under denaturing conditions as suggested by the manufacturer, except that the protein was eluted with a buffer containing only 1 M urea and 250 mM imidazole. A rabbit was injected with 100 μg of the protein emulsified with Freund's complete adjuvant. After three additional booster injections every 4 weeks with Freund's incomplete adjuvant, the rabbit was terminally bled and immunoglobulins G (IgGs) were purified according to standard procedures (14). The serum (no. 2685) was affinity purified by adsorption to antigen immobilized on nitrocellulose (28).

Monoclonal antibodies were produced by immunizing a female BALB/c mouse intracutaneously with 50 μg of CPSF [poly(A)-Sepharose pool (3)] with complete Freund's adjuvant. In intervals of 4 weeks, two booster injections of 50 μg of CPSF emulsified with Freund's incomplete adjuvant were given. After another 3 weeks, 50 μg of CPSF in 50 mM KCl–5 mM Tris-HCl (pH 7.9)–1.5 mM $MgCl_2$–0.1 mM EDTA–0.008% Nonidet P-40 was applied intraperitoneally. Three days later, spleen cells were fused to PAI cells (35) and plated into 10 96-well culture plates containing peritoneal macrophages (15). Hybridomas were screened 12 days later in a dot blot assay with 350 ng of CPSF spotted onto nitrocellulose filters. Positive clones were detected with horseradish peroxidase-coupled goat anti-mouse antibodies and staining with chloronaphthol. Out of 44 positive clones, 2 were subcloned and used for these studies. J1/6 is of the IgG1 subclass and recognizes the 160-kDa subunit of CPSF, whereas J1/27 is of the IgG2a subtype and recognizes the 100-kDa subunit of CPSF as assessed by immunoblotting. Antibodies from 1 liter of tissue culture supernatant were precipitated with $(NH_4)_2SO_4$ at 50% saturation and dialyzed against XTB (50 mM Tris-HCl [pH 7.9], 100 mM KCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol). J1/27 was further purified over protein A-Sepharose according to standard procedures (14).

Immunodepletion of CPSF and inhibition of specific polyadenylation. A total of 20 μl of anti-100-kDa serum or preimmune serum was coupled to protein A-Sepharose equilibrated with IP1 buffer (0.2% bovine serum albumin, 100 mM Na_2HPO_4-HCl [pH 8], 0.1% NaN_3). The resin was washed twice with IP1 and then equilibrated twice with XTB. A total of 50 μl of a Blue Sepharose pool (3) was added and incubated for 3.5 h at 4°C. The supernatant was used for specific polyadenylation assays as described in reference 2, except that the incubation temperature was 30°C. The amounts of depleted fraction used were standardized to total protein concentration and are indicated in the legend to Fig. 3A.

The effect of the anti-100-kDa serum on specific polyadenylation was tested by preincubating assay mixtures containing constant amounts (0.7 U) of CPSF [poly(A)-Sepharose pool (3)] with protein A-Sepharose-purified antiserum or preimmune serum for 5 min on ice. The reactions were then started by the addition of poly(A) polymerase and labeled L3 pre-RNA substrate. The purified antibodies were dialyzed against XTB and had a concentration of 2.2 mg of IgG per ml. The amounts of antibodies added are indicated in the legend to Fig. 3B.

Protein labeling and immunoprecipitations. HeLa cells were grown in Dulbecco minimal essential medium supplemented with 10% fetal calf serum. At 50% confluency, they were washed with phosphate-buffered saline (PBS) and incubated for 15 min with labeling medium (PBS, 10% dialyzed fetal calf serum). Cells were labeled metabolically with 100 μCi of [^{35}S]methionine in 2 ml of labeling medium per 35-mm culture dish overnight at 37°C under a 5% CO_2 atmosphere. The cells were washed twice with ice-cold PBS and scraped off in 1 ml of IP2 buffer (1% Triton X-100, 100 mM Na_2HPO_4-HCl [pH 8], 0.1% NaN_3) containing 40 μg of phenylmethyl-

sulfonyl fluoride per ml. The suspension was triturated 10 times with a 25-gauge needle, incubated for an hour on ice, centrifuged at 10,000 × *g* for 15 min, and frozen at −70°C until used for immunoprecipitation. The equivalent of half a 35-mm plate was used per immunoprecipitation.

Purified CPSF was iodinated with chloramine T (14). CPSF [poly(A)-Sepharose pool (3)] was applied to a preparative Superose 6 column. A total of 1 μg of CPSF of the peak fraction was used for iodination in 200 μl of 250 mM KH_2PO_4 (pH 7.5) with 500 μCi of $Na^{125}I$. The labeling reaction was started by addition of 10 μl of chloramine T (2 mg/ml in 50 mM KH_2PO_4 [pH 7.5]) and stopped after 1 min with 10 μl of $Na_2S_2O_5$ (2 mg/ml in 50 mM KH_2PO_4 [pH 7.5]). The labeled protein was separated from unincorporated label over a Sephadex G-25 column. A total of 5% of the labeling reaction was used per immunoprecipitation.

For immunoprecipitation of CPSF, the antibodies (see figure legends) were coupled to 12 μl of protein A-Sepharose (bed volume) in IP1 buffer (see above) overnight at 4°C. The beads were then washed twice with IP1 and once with IP2. The CPSF-containing sample was incubated with the beads in 0.5 ml of IP2 during 2.5 h at 4°C. The beads were washed three times with buffer IP2 containing 300 mM KCl, once with buffer IP3 (100 mM Na_2HPO_4-HCl [pH 8], 0.1% NaN_3), and once with IP3 diluted 1:10. The beads were resuspended in 20 μl of 2× SDS sample buffer (21) and incubated at 65°C for 20 min. The proteins were separated on an SDS–9% polyacrylamide gel. Proteins labeled with [^{35}S]methionine were detected after fluorography with salicylate by exposure to Kodak X-OMAT AR films. Gels with ^{125}I-labeled proteins were fixed in 20% isopropanol–7.5% acetic acid, dried, and exposed to Fuji medical X-ray films.

UV light-induced cross-linking of HeLa nuclear extract. UV cross-links were essentially done as described in reference 18. Briefly, 10 μl of HeLa nuclear extract (9) was incubated under standard polyadenylation conditions without ATP but with phosphatase inhibitor (5 mM β-glycerophosphate) with 100 fmol of 5′-labeled (33) RNA oligonucleotide (18) for 10 min at 30°C. The reaction mixtures were transferred onto a glass plate on ice and exposed to UV light (254 nm, 2,000 μW/cm^2) for 10 min. CPSF was immunoprecipitated as described above. The proteins were separated on an SDS–9% polyacrylamide gel. The gel was fixed in 7.5% acetic acid–20% isopropanol, dried, and exposed to a Kodak X-OMAT AR film.

Immunofluorescence staining of HeLa cells. Immunolabeling was performed as described previously (5), and the cells were examined in a fluorescence microscope (Polyvar, Reichert-Jung). The photographs were taken on Kodak Elite 400 film.

Nucleotide sequence accession number. The nucleotide sequence has been deposited in the EMBL database under accession number X75931.

RESULTS

Isolation of a cDNA clone encoding the 100-kDa subunit of CPSF. We have used purified CPSF (3) for microsequencing tryptic peptides of the 100-kDa subunit of CPSF. A total of 15 μg of the 100-kDa subunit was digested with trypsin, and the digest was separated by reverse-phase high-pressure liquid chromatography. The amino acid sequences of 11 peptides were determined. Peptide T23-2 (underlined twice in Fig. 2) was used to design a twofold degenerated screening oligonucleotide of 50 nucleotides in length, containing three inosines (22). A single positive clone (A1) of 750 nucleotides resulted from screening 500,000 PFU of a bovine liver cDNA library. A Northern analysis of calf thymus poly(A)$^+$ RNA with clone A1 as a probe revealed strong bands of 4.0 and 2.7 kb and two weak high-molecular-mass species of 6.6 and 8.5 kb (Fig. 1, lane 1). The same probe detected messages in HeLa poly(A)$^+$ RNA that had lengths of 5.6, 4.6, 3.8, and 3.0 kb (lane 2). None of these bands comigrated with a bovine message. A different probe consisting of bovine 3′-noncoding sequences revealed only the bovine 4.0-kb, but no HeLa, message (lanes 3 and 4). This suggests that the coding region, but not the gene organization, is conserved.

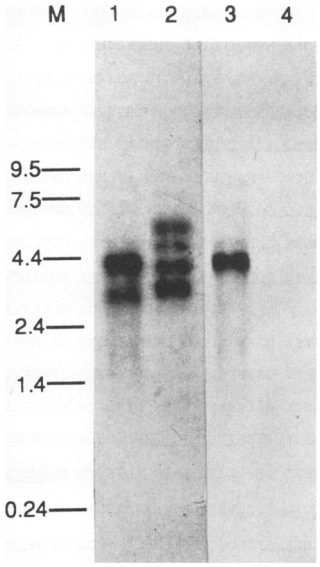

FIG. 1. Northern blot analysis of mRNA encoding the 100-kDa subunit of CPSF. The probe used in lanes 1 and 2 was a cDNA fragment starting at peptide T23 and ending at the stop codon. The probe used for lanes 3 and 4 was the end of the 3.5-kb cDNA clone starting at the *Eco*RI site and containing only 3′-noncoding sequence. Lanes 1 and 3, 10 μg of calf thymus poly(A)$^+$ RNA; lanes 2 and 4, 10 μg of HeLa poly(A)$^+$ RNA. For exact sizes of the bands, see text. The size standards (M) are indicated in kilobases.

Clone A1 was also used as a probe to screen a calf thymus poly(A)$^+$ cDNA library. The largest clone obtained in this screen was 3.5 kb in length. In vitro translation of this cDNA clone in reticulocyte lysate resulted in a protein comigrating with the 100-kDa subunit of purified CPSF. The clone encoded an open reading frame of 782 amino acids but contained no polyadenylation site. The calculated molecular mass of the encoded protein was 88.5 kDa. All 11 peptide sequences could be identified (Fig. 2). The region of the translation initiation codon (AAA AAA *AUG* A) shows only moderate compatibility with the consensus sequence GCC ACC *AUG* G suggested by Kozak (19). The most critical base, the A at position −3, is present. Upstream of the AUG codon, each of the three reading frames has at least one stop codon. No homologous sequences or known sequence motifs were found by searching the most common databases. In particular, the sequence contains no RNA binding motifs or sequences resembling a nuclear localization signal, although the 100-kDa subunit appears to be a nuclear protein (see below). The protein is highly charged: it contains 31% charged amino acids with an even distribution. Particularly striking is that it contains 40% more acidic residues than an average protein (22), resulting in a calculated pI of 4.8.

A cDNA fragment starting with peptide T23-2 and ending at the stop codon was amplified with *Pyrococcus furiosus* (*Pfu*)

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  1  MTSIIKLTTL SGVQEESALC YLLQVDEFRF LLDCGWDEHF   40
 41  SMDIIDSLRK HVHQIDAVLL SHPDPLHLGA LPYAVGKLGL   80
 81  NCAIYATIPV YKMGQMFMYD LYQSRHNTED FTLFTLDDVD  120
121  AAFDKIQQLK FSQIVNLKGK GHGLSITPLP AGHMIGGTIW  160
161  KIVKDGEEEI VYAVDFNHKR EIHLNGCSLE MLSRPSLLIT  200
201  DSFNATYVQP RRKQRDEQLL TNVLETLRGD GNVLIAVDTA  240
241  GRVLELAQLL DQIWRTKDAG LGVYSLALLN NVSYNVVEFS  280
281  KSQVEWMSDK LMRCFEDKRN NPFQFRHLSL CHGLSDLARV  320
321  PSPKVVLASQ PDLECGFSRD LFIQWCQDPK NSIILTYRTT  360
361  PGTLARFLID NPSEKVTEIE LRKRVKLEGK ELEEYLEKEK  400
401  LKKEAAKKLE QSKEADIDSS DESDAEEDID QPSAHKTKHD  440
441  LMMKGEGSRK GSFFKQAKKS YPMFPAPEER IKWDEYGEII  480
481  KPEDFLVPEL QATEEEKSKL ESGLTNGDEP MDQDLSDVPT  520
521  KCISTTESIE IKARVTYIDY EGRSDGDSIK KIINQMKPRQ  560
561  LIIVHGPPEA SQDLAECCRA FGGKDIKVYM PKLHETVDAT  600
601  SETHIYQVRL KDSLVSSLQF CKAKDAELAW IDGVLDMRVS  640
641  KVDTGVILEE GELKDDGEDS EMQVDAPSDS SVIAQQKAMK  680
681  SLFGDDEKET GEESEIIPTL EPLPPHEVPG HQSVFMNEPR  720
721  LSDFKQVLLR EGIQAEFVGG VLVCNNQVAV RRTETGRIGL  760
761  EGCLCQDFYR IRDLLYEQYA IV                     782
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FIG. 2. Predicted amino acid sequence of the CPSF 100-kDa subunit. The 2,346-bp-long open reading frame of cDNA clone 26/2 was translated into a protein sequence of 782 amino acids. Underlined amino acids correspond to the tryptic fragments that were sequenced. The peptide sequence underlined twice (T23-2) was used to design a screening oligonucleotide.

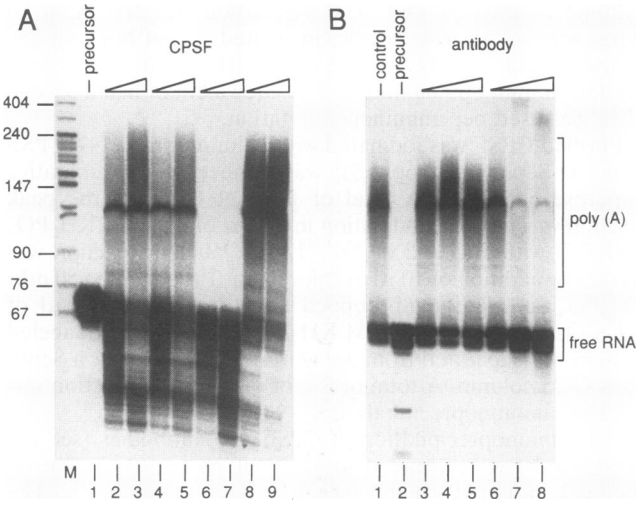

FIG. 3. Clone 26/2 codes for the 100-kDa subunit of CPSF. Polyadenylation assays contained 60 fmol of uniformly ^{32}P-labeled, precleaved L3 RNA and indicated amounts of CPSF. The reaction products were separated over a denaturing 12% polyacrylamide gel. (A) Immunodepletion assay. Lane 1, precleaved L3 precursor RNA. The following amounts of CPSF were used: lanes 2 and 3, 0.1 and 0.2 μl of an untreated Blue Sepharose pool; lanes 4 and 5, 0.2 and 0.4 μl of preimmune serum-depleted Blue Sepharose pool; lanes 6 and 7, 0.2 and 0.4 μl of the Blue Sepharose pool immunodepleted with antibody no. 2685 against the 100-kDa CPSF subunit; lanes 8 and 9, rescue of the polyadenylation activity in reaction 7; 4 and 8 U of pure CPSF [poly(A)-Sepharose pool] was added back. M indicates the positions of DNA length markers in bases. (B) Inhibition of polyadenylation with anti-100-kDa subunit antiserum. To complete polyadenylation reactions containing 0.7 U of CPSF, increasing amounts of protein A-Sepharose-purified preimmune serum or serum no. 2685 against the 100-kDa CPSF subunit were added. Lane 1, control reaction without addition of any antibody; lane 2, precleaved L3 RNA; lanes 3 to 5, preincubation of the reaction with 22 ng, 220 ng, and 2.2 μg of IgG from preimmune serum; lanes 6 to 8, preincubation of the reaction with 22 ng, 220 ng, and 2.2 μg of IgG from the serum against the 100-kDa subunit of CPSF. Poly(A) indicates the polyadenylated reaction products.

DNA polymerase and cloned into a His-tag expression vector. The resulting protein fragment of 32 kDa, corresponding to the C-terminal third of the 100-kDa subunit of CPSF, was expressed in *E. coli*, purified, and used to generate rabbit polyclonal antibodies. The serum specifically recognized the 100-kDa subunit in enriched and pure bovine CPSF preparations as well as in HeLa nuclear extracts as assessed by immunoblotting (not shown). The antibodies were used to confirm the identity of the cDNA clone by functional assays. To deplete a crude fraction of CPSF activity, preimmune serum and serum against the expressed polypeptide were bound to protein A-Sepharose and incubated with 50 μl of partially purified CPSF (Blue Sepharose pool [3]). The supernatant was tested for specific polyadenylation activity after complementation with purified poly(A) polymerase (Fig. 3A). The sample treated with the serum against the 100-kDa subunit was completely devoid of any CPSF activity (lanes 6 and 7), whereas the sample depleted with preimmune serum still showed CPSF activity comparable to that of the untreated fraction (lanes 4 and 5 and 2 and 3, respectively). This effect was due to the depletion of CPSF since the depleted activity could be restored by addition of purified CPSF [poly(A)-Sepharose pool, lanes 8 and 9]. In a second experiment, pure CPSF was preincubated with increasing concentrations of protein A-Sepharose-purified immunoglobulins from preimmune serum and anti-100-kDa subunit serum and then used in standard polyadenylation assays. The reactions were started by the addition of labeled precursor RNA and poly(A) polymerase. Lanes 6 to 8 in Fig. 3B show an antibody concentration-dependent inhibition of the polyadenylation reaction, whereas the preimmune serum did not influence the reaction (lanes 3 to 5). A total of 220 ng of IgG inhibited the polyadenylation reaction by 50% compared with the control reaction.

Composition of CPSF. To further characterize CPSF, we raised monoclonal antibodies against native CPSF in mice. Two cell lines secreting antibodies against the 160-kDa (J1/6) and 100-kDa (J1/27) subunits of CPSF were subcloned, and the antibodies were collected from the tissue culture supernatant.

CPSF has been purified from calf thymus extracts and HeLa cell nuclear extracts. Bienroth et al. (3) purified the protein as a heterotetramer consisting of subunits of 160, 100, 73, and 30 kDa. Murthy and Manley (27) purified CPSF as a heterotrimer lacking the 30-kDa subunit. To determine the composition of CPSF in vivo, we metabolically labeled HeLa cell monolayers with [^{35}S]methionine (see Materials and Methods). After washing, the cells were lysed with Triton X-100, and CPSF was immunoprecipitated with polyclonal and monoclonal antibodies. Lane 2 in Fig. 4A shows the labeled proteins precipitated from the lysate with affinity-purified serum raised against the C-terminal portion of the 100-kDa subunit of CPSF. Clearly, proteins of 160, 100, and 73 kDa were coprecipitated. In addition, on the original autoradiograph a weakly labeled protein which migrated at the position of the 30-kDa molecular mass marker was also visible. Figure 3B shows an overex-

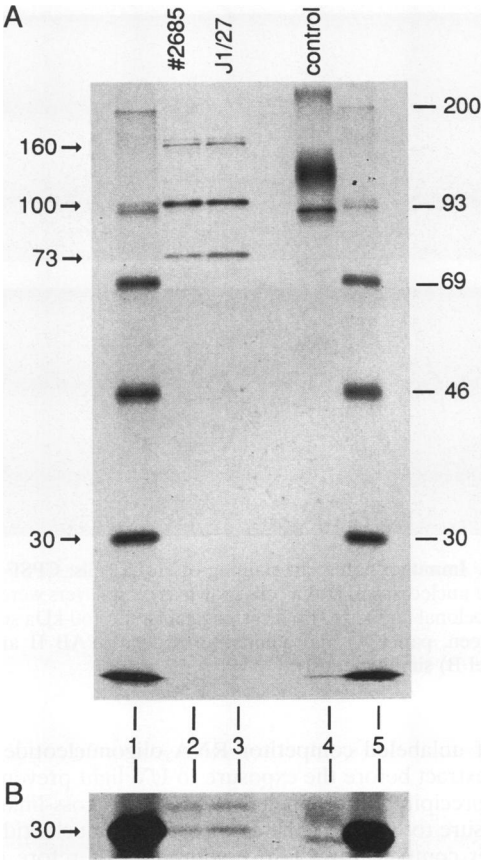

FIG. 4. Monoclonal and polyclonal antibodies against the 100-kDa CPSF subunit coprecipitate the four CPSF subunits. [^{35}S]methionine-labeled HeLa cells were extracted with Triton X-100, and CPSF was immunoprecipitated. The precipitated proteins were separated on an SDS–9% polyacrylamide gel and visualized by fluorography. Proteins of 160, 100, 73, and 30 kDa are coprecipitated. (A) Lanes 1 and 5, ^{14}C-labeled molecular weight markers (weights given in thousands at right); lane 2, immunoprecipitation with affinity-purified serum no. 2685 against the CPSF 100-kDa subunit; lane 3, immunoprecipitation with the monoclonal antibody J1/27 directed against the CPSF 100-kDa subunit; lane 4, immunoprecipitation with a monoclonal antibody against human NaK ATPase α-subunit. (B) Portion of the same gel around the 30-kDa marker. The lanes correspond to those in panel A. The 30-kDa band is indicated by an arrow.

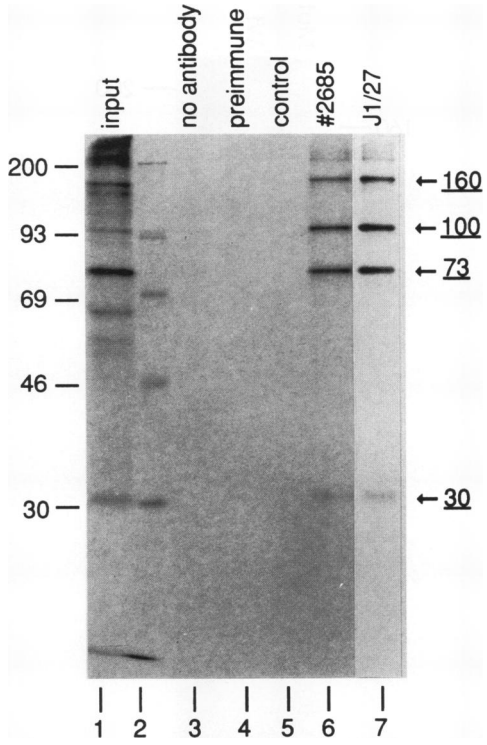

FIG. 5. Antibodies against CPSF coprecipitate four polypeptides of 160, 100, 73, and 30 kDa from an iodinated CPSF fraction. Highly pure CPSF (Superose 6 peak fraction) was iodinated and immunoprecipitated. The precipitated proteins were separated over an SDS–9% polyacrylamide gel and visualized by autoradiography. The positions of the CPSF subunits are indicated by their molecular weights (in thousands) and arrows. Lane 1, labeled CPSF fraction without immunoprecipitation; lane 2, ^{14}C-labeled molecular weight markers (weights given in thousands at left); lane 3, immunoprecipitation without antibody; lane 4, immunoprecipitation with rabbit preimmune serum; lane 5, immunoprecipitation with a control monoclonal antibody directed against splicing factor SF3a^{66}; lane 6, immunoprecipitation with rabbit serum no. 2685 against the 100-kDa CPSF subunit; lane 7, immunoprecipitation with monoclonal antibody J1/27 directed against the 100-kDa CPSF subunit. The precipitated bands larger than 200 kDa are not present in all CPSF preparations.

posure of the region of the 30-kDa marker of the same gel. The 30-kDa subunit in pure CPSF preparations shows the same migration behavior (see also Fig. 5). As seen in lane 3, the monoclonal antibody J1/27, recognizing the 100-kDa subunit, precipitated proteins with the same molecular masses. A control monoclonal antibody against human NaK ATPase α-subunit (14a) did not precipitate proteins corresponding to CPSF (lane 4). In addition, on overexposed films bands of 68 and 35 kDa were seen. The 35-kDa protein could be the U1 small nuclear ribonucleoprotein particle-specific U1A protein (see Discussion). The protein pattern did not change when more stringent washing conditions of up to 600 mM KCl were used. The major contaminants of CPSF preparations of 63 and 140 kDa reported by Bienroth et al. (3) do not coprecipitate. The double band seen at 160 kDa is probably due to protein degradation, since it was not found in every immunoprecipitation.

It was not clear whether the weak band at 30 kDa was the 30-kDa subunit of CPSF and whether the minor band seen at 35 kDa was also associated with CPSF. We therefore further purified CPSF [poly(A)-Sepharose pool (3)] over a Superose 6 gel filtration column and iodinated the proteins of the peak fraction with chloramine T. Immunoprecipitation of CPSF from the labeled fraction with either affinity-purified antiserum or the monoclonal antibody J1/27 coprecipitated all four subunits of CPSF including the 30-kDa protein (Fig. 5, lanes 6 and 7). No proteins were precipitated when protein A-Sepharose alone, preimmune serum, or a monoclonal antibody against splicing factor SF3a^{66} (4) was used (lanes 3 to 5). The precipitation was specific, since not all proteins present in the fraction were precipitated (lane 1). The same pattern was observed after labeling and precipitation of two independent CPSF preparations. The 30-kDa protein therefore behaves like a genuine subunit of CPSF.

The 160- and 30-kDa subunits of CPSF can be cross-linked to RNA. Under polyadenylation conditions, proteins of 160, 130, 64, and 35 kDa can be cross-linked by UV light in nuclear extracts or crude fractions to RNA substrates containing a functional polyadenylation signal (12, 18, 25, 47). Except for

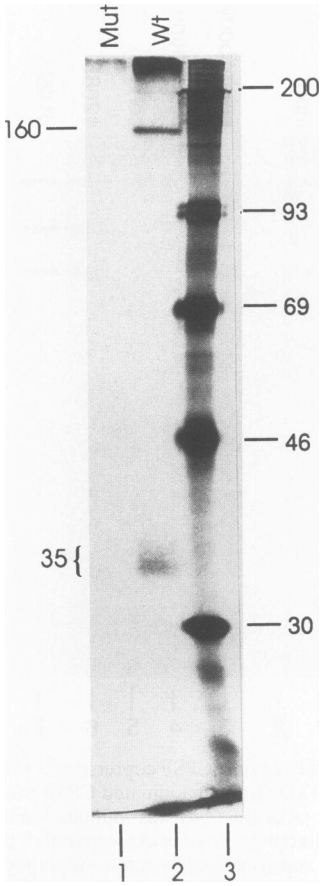

FIG. 6. The 160- and 30-kDa subunits of CPSF can be UV cross-linked to RNA oligonucleotides in HeLa nuclear extract. 5′-labeled RNA oligonucleotides were incubated with HeLa nuclear extract and exposed to UV light. CPSF was immunoprecipitated with monoclonal antibody J1/27. The positions of the 160- and 30-kDa CPSF subunits are indicated. Lane 1, immunoprecipitated proteins cross-linked to the oligonucleotide containing a mutant (AAGAAA) polyadenylation signal; lane 2, immunoprecipitated proteins cross-linked to the oligonucleotide containing the wild-type polyadenylation signal (AAUAAA); lane 3, ^{14}C-labeled molecular weight markers (weights given in thousands at right).

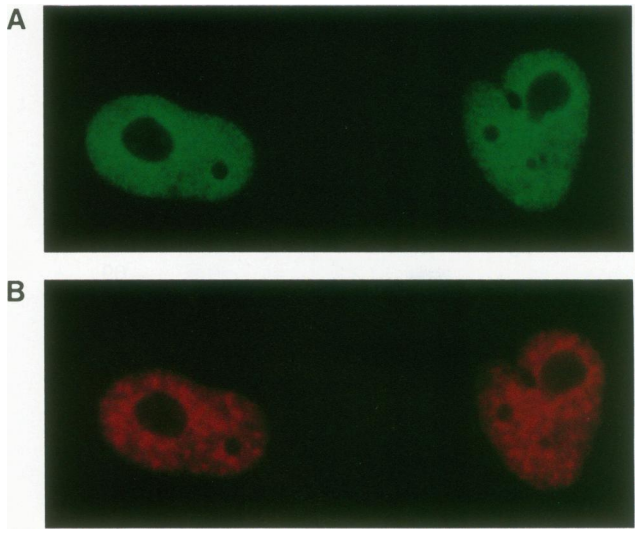

FIG. 7. Immunofluorescent staining of HeLa cells: CPSF is localized in the nucleoplasm. HeLa cells grown as monolayers were labeled with monoclonal antibody J1/6 directed against the 160-kDa subunit of CPSF (green, panel A) and affinity-purified anti-PAB II antiserum (red, panel B) simultaneously.

the protein of 64 kDa, which was shown to be a subunit of the cleavage stimulation factor CStF (37), the identity of the other cross-linked proteins has not been proven, although it was suggested that the 160- and 35-kDa cross-links belonged to CPSF (12, 18, 25). The 130-kDa band also cofractionated with CPSF activity (12). We cross-linked labeled RNA oligonucleotides of 18 nucleotides in length, containing either a wild-type or a mutant polyadenylation signal, to proteins of HeLa nuclear extracts and immunoprecipitated CPSF with the monoclonal antibody J1/27. Lane 2 in Fig. 6 shows that the 160- and 30-kDa subunits of CPSF had been labeled by cross-linking to the wild-type RNA oligonucleotide. The change of the migration behavior of the 30-kDa subunit observed here was also described by Keller et al. (18) and was attributed to the increase in molecular weight after binding of the RNA (5 kDa). Lane 1 shows that the cross-link depended on a functional polyadenylation signal. The same cross-linked proteins were immunoprecipitated by the serum raised against the 100-kDa subunit or the monoclonal antibody J1/6 directed against the 160-kDa subunit of CPSF. Addition of a 400-fold excess of unlabeled competitor RNA oligonucleotide to the nuclear extract before the exposure to UV light prevented the immunoprecipitation of labeled CPSF-RNA cross-links. After the exposure to UV light, the cold RNA oligonucleotide could no longer compete for CPSF binding and therefore did not prevent the immunoprecipitation of the two UV-cross-linked proteins (not shown). Although we are confident that the 160-kDa band is due to RNA-protein cross-linking of the largest subunit of CPSF, the assignment of the 35-kDa UV cross-link to the 30-kDa subunit of CPSF is less certain. We cannot exclude the possibility that this cross-link is due to U1A protein associated with CPSF (see Discussion).

Nuclear localization of CPSF. The cellular distribution of CPSF was examined by indirect immunofluorescence microscopy. HeLa cells were stained with the monoclonal antibody J1/6 recognizing the 160-kDa subunit of CPSF and, simultaneously, with affinity-purified polyclonal antibodies against PAB II (20). CPSF was restricted to the nucleoplasm and not present in the nucleoli (Fig. 7A). It showed a uniformly distributed, fine, granular staining pattern. In contrast to PAB II (Fig. 7B), it was not concentrated in nuclear speckles, which were reported to contain factors involved in pre-mRNA splicing (5, 10). The pattern did not change when the cells were stained with the monoclonal antibody J1/27 detecting the 100-kDa subunit of CPSF (not shown). The staining pattern is reminiscent of the pattern described for the 64-kDa subunit of CStF (37). CPSF and CStF are both involved in the first step of polyadenylation and therefore are expected to show an overlapping localization.

DISCUSSION

We have isolated a cDNA clone coding for the bovine 100-kDa subunit of the cleavage and polyadenylation specificity factor CPSF. All 11 tryptic peptides sequenced were found in the open reading frame of 782 amino acids. The predicted molecular mass of 88.5 kDa is too small compared with the migration behavior seen on SDS-polyacrylamide gels. How-

ever, the 100-kDa subunit expressed in *E. coli* or made in an in vitro translation system comigrated with the 100-kDa subunit of purified CPSF. We showed that an antiserum raised against the C terminus of the subunit could deplete a partially purified CPSF fraction of CPSF activity. The antiserum also inhibited the specific polyadenylation of precleaved RNA substrates.

Polyclonal antibodies against the C-terminal third of the 100-kDa subunit of CPSF recognized this subunit in CPSF purified from calf thymus on Western blots (immunoblots). The antiserum against the bovine polypeptide cross-reacts with CPSF in HeLa nuclear extract and in *Xenopus laevis* (3a). This antiserum inhibits the specific polyadenylation in vitro (Fig. 3B) but has no effect on the cleavage reaction (not shown). Complex formation between CPSF and RNA or CPSF, poly(A) polymerase, and RNA is not inhibited. Rather, on native gels the polyadenylation complexes are further retarded by the antiserum (24a). The monoclonal antibody J1/27 recognizing the same subunit of CPSF inhibits the cleavage reaction but not polyadenylation (1a). The determination of the epitope recognized by the antibody might lead to the identification of an important site involved specifically in the cleavage but not the polyadenylation reaction.

CPSF has been described as a factor consisting of four (160, 100, 73, and 30 kDa [3]) or three (165, 105, and 70 kDa [27]) subunits. It was suggested that the additional 30-kDa subunit was only loosely associated with CPSF (24) and might be dispensable for the activity in vitro (43). Immunoprecipitation of metabolically labeled CPSF from whole-cell extracts clearly showed the coprecipitation of the 160-, 100-, and 73-kDa subunits and probably also the 30-kDa subunit. The pattern of immunoprecipitated proteins was identical with polyclonal or monoclonal antibodies against the 100-kDa subunit or a monoclonal antibody against the 160-kDa subunit. Further purification of CPSF over a Superose 6 gel filtration column showed the copurification of the 30-kDa subunit of CPSF together with the three large subunits as judged by Coomassie blue-stained protein gels (not shown). Immunoprecipitation of CPSF from the ^{125}I-labeled peak fraction of the Superose 6 column precipitated the 30-kDa protein together with the 160-, 100-, and 73-kDa subunits. This shows that the four polypeptides form a tight complex. No additional proteins are coprecipitated. We cannot exclude the possibility that the 30-kDa subunit is a degradation product of the 160-kDa subunit. However, neither the immunoprecipitation of ^{35}S-labeled CPSF nor that of iodinated CPSF revealed a 130-kDa component or any other degradation products which could be the counterparts of the 30-kDa polypeptide.

Moore et al. (25) and Gilmartin and Nevins (12) have reported that a protein of 160 kDa can be cross-linked to RNA containing a functional polyadenylation signal. Other investigators have not detected this UV cross-link (37, 47). Keller et al. (18) have detected the same UV cross-link and an additional one at 35 kDa, both of which depended on a functional polyadenylation site and copurified with CPSF. These cross-links were immunoprecipitated with antibodies directed against the 100- or the 160-kDa subunits of CPSF. The cross-link at 130 kDa in crude CPSF (at that time called PF2) fractions described by Gilmartin and Nevins (12) was not coprecipitated.

The U1 small nuclear ribonucleoprotein particle has been suggested to participate in different aspects of the 3′-processing reaction (23, 44). Its protein subunit U1A regulates its own polyadenylation (13). Different preparations of purified CPSF show variable amounts of U1A protein as assessed by Western blot analysis with a U1A-specific antiserum (17a). This protein migrates with a slightly higher molecular weight than that of the 30-kDa subunit of CPSF on SDS-polyacrylamide gels. Most of it can be removed from purified CPSF by gel filtration over a Superose 6 column, where it is clearly retarded compared with all four CPSF subunits. In addition, we have obtained peptide sequences of the 30-kDa subunit of CPSF which show no homology to the U1A protein. Lutz and Alwine (23) reported the cross-linking of the U1A protein to upstream efficiency elements of the simian virus 40 late polyadenylation site. This cross-link depends on the presence of U-rich upstream efficiency elements which are located between 15 and 50 nucleotides upstream of the polyadenylation signal. Although the molecular mass of U1A is 35 kDa, it is not likely that it is responsible for the 35-kDa cross-link we see, because our oligonucleotide of 18 bp does not contain any sequence similar to an upstream efficiency element. We therefore attribute the two UV cross-links to the 160- and 30-kDa subunits of CPSF.

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REFERENCES

1. **Bardwell, V. J., M. Wickens, S. Bienroth, W. Keller, B. S. Sproat, and A. I. Lamond.** 1991. Site-directed ribose methylation identifies 2′-OH groups in polyadenylation substrates critical for AAUAAA recognition and poly(A) addition. Cell **65**:125–133.

1a. **Beyer, K., and A. Jenny.** Unpublished data.

2. **Bienroth, S., W. Keller, and E. Wahle.** 1993. Assembly of a processive messenger RNA polyadenylation complex. EMBO J. **12**:585–594.

3. **Bienroth, S., E. Wahle, C. Suter-Crazzolara, and W. Keller.** 1991. Purification and characterisation of the cleavage and polyadenylation specificity factor involved in the 3′ processing of messenger RNA precursors. J. Biol. Chem. **266**:19768–19776.

3a. **Bilger, A., and M. Wickens.** Personal communication.

4. **Brosi, R., H. Hauri, and A. Krämer.** 1993. Separation of splicing factor SF3 into two components and purification of SF3a activity. J. Biol. Chem. **268**:17640–17646.

5. **Carmo-Fonseca, M., D. Tollervey, R. Pepperkok, S. M. Barabino, A. Merdes, C. Brunner, P. D. Zamore, M. R. Green, E. Hurt, and A. I. Lamond.** 1991. Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery. EMBO J. **10**:195–206.

6. **Christofori, G., and W. Keller.** 1988. 3′ cleavage and polyadenylation of mRNA precursors in vitro requires a poly(A) polymerase, a cleavage factor, and a snRNP. Cell **54**:875–889.

7. **Christofori, G., and W. Keller.** 1989. Poly(A) polymerase purified from HeLa cell nuclear extract is required for both cleavage and polyadenylation of pre-mRNA in vitro. Mol. Cell. Biol. **9**:193–203.

8. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.

9. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1488.

10. **Fu, X.-D., and T. Maniatis.** 1990. A factor required for mammalian spliceosome assembly is localised to discrete regions in the nucleus. Nature (London) **343**:437–441.

11. **Gallie, D. R.** 1991. The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev. **5**:2108–2116.

12. **Gilmartin, G. M., and J. R. Nevins.** 1989. An ordered pathway of assembly of components required for polyadenylation site recognition and processing. Genes Dev. **3:**2180–2189.
13. **Gunderson, S. J., K. Beyer, G. Martin, W. Keller, W. C. Boelens, and I. W. Mattai.** 1994. The human U1A snRNP protein regulates polyadenylation via a direct interaction with poly(A) polymerase. Cell **76:**531–541.
14. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14a. **Hauri, H.-P.** Unpublished data.
15. **Hauri, H. P., E. E. Sterchi, D. Bienz, J. A. M. Fransen, and A. Marxer.** 1985. Expression and intracellular transport of microvillus membrane hydrolases in human intestinal epithelial cells. J. Cell Biol. **101:**838–851.
16. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
17. **Jackson, R. J., and N. Standard.** 1990. Do the poly(A) tail and 3′ untranslated region control mRNA translation? Cell **62:**15–24.
17a. **Jenny, A., and E. Wahle.** Unpublished data.
18. **Keller, W., S. Bienroth, K. M. Lang, and G. Cristofori.** 1991. Cleavage and polyadenylation factor CPF specifically interacts with the pre-mRNA 3′ processing signal AAUAAA. EMBO J. **10:**4241–4249.
19. **Kozak, M.** 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. **266:**19867–19870.
20. **Krause, S., S. Fakan, K. Weis, and E. Wahle.** 1994. Immunodetection of poly(A) binding protein II in the cell nucleus. Exp. Cell Res. **214:**75–82.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Lathe, R.** 1985. Synthetic oligonucleotide probes deduced from amino acid sequence data. J. Mol. Biol. **183:**1–12.
23. **Lutz, C. S., and J. C. Alwine.** 1994. Direct interaction of the U1 snRNP-A protein with the upstream efficiency element of the SV40 late polyadenylation signal. Genes Dev. **8:**576–586.
24. **Manley, J., and N. J. Proudfoot.** 1994. RNA 3′ ends. Formation and function—meeting review. Genes Dev. **8:**259–264.
24a. **Martin, G., and A. Jenny.** Unpublished data.
25. **Moore, C. L., J. Chen, and J. Whoriskey.** 1988. Two proteins crosslinked to RNA containing the adenovirus L3 poly(A) site require the AAUAAA sequence for binding. EMBO J. **7:**3159–3169.
26. **Moore, C. L., and P. A. Sharp.** 1985. Accurate cleavage and polyadenylation of exogenous RNA substrate. Cell **41:**845–855.
27. **Murthy, K. G. K., and J. L. Manley.** 1992. Characterisation of the multisubunit cleavage-polyadenylation specificity factor from calf thymus. J. Biol. Chem. **267:**14804–14811.
28. **Olmstedt, J. B.** 1981. Affinity purification of antibodies from diazotised paper blots of heterogeneous protein samples. J. Biol. Chem. **256:**11955–11957.
29. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
30. **Proudfoot, N.** 1991. Poly(A) signals. Cell **64:**671–674.
31. **Sachs, A., and E. Wahle.** 1993. Poly(A) tail metabolism and function in eukaryotes. J. Biol. Chem. **31:**2295–2298.
32. **Sachs, A. B.** 1993. Messenger RNA degradation in eukaryotes. Cell **74:**413–421.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Sheets, M. D., and M. Wickens.** 1989. Two phases in the addition of a poly(A) tail. Genes Dev. **3:**1401–1412.
35. **Stocker, and T. Staehelin.** 1982. Generation of two new mouse myeloma cell lines "PAI" and "PAI-0" for hybridoma production. Res. Disclosure **May:**154–157.
36. **Takagaki, Y., and J. L. Manley.** 1992. A human polyadenylation factor is a G protein β-subunit homologue. J. Biol. Chem. **267:**23471–23474.
37. **Takagaki, Y., J. L. Manley, C. C. MacDonald, J. Wilusz, and T. Shenk.** 1990. A multisubunit factor, CStF, is required for polyadenylation of mammalian pre-mRNAs. Genes Dev. **4:**2112–2120.
38. **Takagaki, Y., L. C. Rhyner, and J. L. Manley.** 1989. Four factors are required for 3′-end cleavage of pre-mRNAs. Genes Dev. **3:**1711–1724.
39. **Ullrich, A., C. H. Berman, T. J. Dull, A. Gray, and J. M. Lee.** 1984. Isolation of the human insulin-like growth factor I gene using a synthetic DNA probe. EMBO J. **3:**361–364.
40. **Wahle, E.** 1991. A novel poly(A)-binding protein acts as a specificity factor in the second phase of messenger RNA polyadenylation. Cell **66:**759–768.
41. **Wahle, E.** 1991. Purification and characterisation of a mammalian polyadenylate polymerase involved in the 3′ end processing of messenger RNA precursors. J. Biol. Chem. **226:**3131–3139.
42. **Wahle, E., and W. Keller.** 1992. The biochemistry of 3′-end cleavage and polyadenylation of messenger RNA precursors. Annu. Rev. Biochem. **61:**419–440.
43. **Wahle, E., and W. Keller.** 1993. RNA-protein interactions in mRNA 3′-end formation. Mol. Biol. Rep. **18:**157–161.
44. **Wassarman, K. M., and J. A. Steitz.** 1993. Association with terminal exons in pre-mRNAs: a new role for the U1 snRNP? Genes Dev. **7:**647–659.
45. **Wickens, M.** 1990. How the messenger got its tail: addition of poly(A) in the nucleus. Trends Biochem. Sci. **15:**277–281.
46. **Wickens, M.** 1992. Forward, backward, how much, when: mechanisms of poly(A) addition and removal and their role in early development. Dev. Biol. **3:**399–412.
47. **Wilusz, J., and T. Shenk.** 1988. A 64kD nuclear protein binds to RNA segments that include the AAUAAA polyadenylation motif. Cell **52:**221–228.
48. **Wilusz, J., T. Shenk, Y. Takagaki, and J. L. Manley.** 1990. A multicomponent complex is required for AAUAAA-dependent cross-linking of a 64-kilodalton protein to polyadenylation substrates. Mol. Cell. Biol. **10:**1244–1248.